Disulfiram is a direct and potent inhibitor of human O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) in brain tumor cells and mouse brain and markedly increases the alkylating DNA damage

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The alcohol aversion drug disulfiram (DSF) reacts and conjugates with the protein-bound nucleophilic cysteines and is known to elicit antitumor effects alone or improve the efficacy of many cancer drugs. We investigated the effects of DSF on human O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein and chemotherapy target that removes the mutagenic O\textsuperscript{6}-alkyl groups from guanines, and thus confers resistance to alkylating agents in brain tumors. We used DSF, copper-chelated DSF or CuCl\textsubscript{2}-DSF combination and found that all treatments inhibited the MGMT activity in two brain tumor cell lines in a rapid and dose-dependent manner. The drug treatments resulted in the loss of MGMT protein from tumor cells through the ubiquitin-proteasome pathway. Evidence showed that Cys145, a reactive cysteine, critical for DNA repair was the sole site of DSF modification in the MGMT protein. DSF was a weaker inhibitor of MGMT, compared with the established O\textsuperscript{6}-benzylguanine; nevertheless, the 24–36h suppression of MGMT activity in cell cultures vastly increased the alkylation-induced DNA interstrand cross-linking, G\textsubscript{2}/M cell cycle blockade, cytotoxicity and the levels of apoptotic markers. Normal mice treated with DSF showed significantly attenuated levels of MGMT activity and protein in the liver and brain tissues. In nude mice bearing T98 glioblastoma xenografts, there was a preferential inhibition of tumor MGMT. Our studies demonstrate a strong and direct inhibition of MGMT by DSF and support the repurposing of this brain penetrating drug for glioma therapy. The findings also imply an increased risk for alkylation damage in alcoholic patients taking DSF.

Introduction

O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) is a unique antimutagenic DNA repair protein that plays a crucial role in the defense against alkylating agents, particularly those that generate the O\textsuperscript{6}-alkylguanines (1,2). Guanine is the most preferred base for alkylation, and the adducts at the O\textsuperscript{6}-guanine are particularly critical, because the O\textsuperscript{6}-alkylguanines pair aberrantly with thymine, resulting in GC to AT transitions (3). MGMT repairs O\textsuperscript{6}-alkylguanine and O\textsuperscript{4}-alkylthymine lesions by transferring the alkyl groups to an active site cysteine residue (Cys145) in the protein in a stoichiometric and suicidal reaction, so that the guanine in the DNA is simply restored in an error-free direct reversal reaction (2). Because, the alkyl group is covalently bound to the protein, MGMT is functionally inactivated after each reaction, and the inactive protein is degraded through the ubiquitin (ub) proteolytic pathway (4). MGMT is abundantly expressed in liver and other normal tissues, but is present at very low levels in the bone marrow and normal brain (5). The repair function of MGMT is essential for the removal of O\textsuperscript{6}-guanine alkylations introduced by the carcinogens present in cooked meat, endogenous metabolites such as the S-adenosylmethionine, nitrosated amino acids and tobacco smoke (6), and maintaining genomic stability.

MGMT appears to have a strong linkage with another public health problem, namely the chronic alcohol abuse and the resulting pathological effects in liver and brain (7) as well. A number of studies have described the suppression of MGMT and an increased alkylation damage following acute or chronic alcohol intake (7–10). Disulfiram (DSF, bis-diethylthiocarbamoyl disulfide), also known as Antabuse, is a carabamate derivative clinically used for treating alcoholism and more recently for cocaine addiction (11,12). DSF is a relatively non-toxic substance when administered alone, but markedly alters the metabolism of alcohol by irreversibly inhibiting the hepatic aldehyde dehydrogenase (ALDH) and causing an accumulation of acetdehyde and consequent aversion to further drinking (11). DSF and its metabolites form mixed disulfide bridges with a critical cysteine (Cys302) near the active site region of ALDH (13) to inactivate the enzyme. Similarly, the reactive cysteines 179 and 234 in the ub-activating enzyme E1 are targeted by DSF for conjugation (14). Recently, we showed that DSF reacts similarly with a number of redox-sensitive proteins such as the p53 tumor suppressor, NF-kB and ub-activating enzyme E1 and lead to their degradation (15).

MGMT is highly expressed in about 80% of brain tumors and other cancers (16). Paradoxically, its antimutagenic function interferes with the cytotoxic actions of anticancer alkylating agents (16,17). This is because MGMT effectively repairs the O\textsuperscript{6}-methylguanine and O\textsuperscript{4}-chloroethylguanine lesions induced by methylating agents [temozolomide (TMZ), dacarbazine and procarbazine] and chloroethylating agents [1,3-bis-2-chloroethyl-1-nitrosourea (BCNU) and CCNU], respectively, thereby preventing the generation of mutagenic lesions and interstrand DNA cross-links. Consequently, MGMT has emerged as a central determinant of tumor resistance to alkylating agents.

In view of this therapeutic relevance, MGMT has been extensively targeted for inhibitor development. Much success has been achieved through the design of psuedosubstrate inhibitors, namely the O\textsuperscript{6}-benzylguanine (BG) and O\textsuperscript{4}-[4-bromoethyl]guanine (Patrin-2), which are currently undergoing clinical trials (17,18). In this biochemical strategy, the free base inhibitors (BG) are first administered to inhibit MGMT and create a DNA repair-deficient state followed by alkylating agents to increase the DNA damage and antitumor efficacy. BG is a specific and powerful inhibitor of MGMT and causes a prolonged suppression of DNA repair (48–72h) in cultured tumor cells (19). Although this approach has shown a positive outcome in cultured cells and xenograft settings (17,18), a significant drawback is the excess of bone marrow toxicity encountered in patients enrolled in BG + alkylating agent combination regimens. Hematopoietic stem cells contain very low levels of MGMT, whose inactivation by BG predisposes them to excessive alkylation damage, which results in therapy discontinuance and necessitates the use of alkylating drugs at sub-therapeutic levels. This problem has prompted a gene therapy approach involving the transduction of BG-resistant MGMT genes (G156A or P140K) into the hematopoietic stem cells (20). However, the cost, complexity and safety issues make this approach cumbersome and impractical.

Abbreviations: ALDH, aldehyde dehydrogenase; ATP, adenosine triphosphate; BCNU, 1,3-bis-2-chloroethyl-1-nitrosourea; BG, O\textsuperscript{6}-benzylguanine; CuDSF, copper-chelated disulfiram; DSF, disulfiram; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; MGMT, O\textsuperscript{6}-methylguanine-DNA methyltransferase; MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PS-341, bortezomib; rMGMT, recombinant MGMT; TMZ, temozolomide; ub, ubiquitin.
The considerations above justify an urgent need for new and transient inhibitors for human MGMT. In our attempts to design better and rational inhibitors for MGMT, we exploited the reactivity of Cys145, which accepts the alkyl residues in the self-inactivating reaction. This active site cysteine has a low pK\textsubscript{a} of 4.5 (21) and reacts readily with glutathione (GSH) forming a mixed disulfide linkage (22). Cysteine 145 of MGMT is also a good substrate for nitrosylation (23). Therefore, we hypothesized that drugs/small molecules with strong affinity for reactive cysteines will be able to bind and inactivate the MGMT protein. Our efforts showed that DSF is such a candidate and this report describes a detailed characterization of MGMT inhibition by DSF, the augmented alkylation damage and a synergistic cytotoxicity. The significance of our findings for increased carcinogenic risk in alcoholic patients taking DSF is also discussed.

Materials and methods

Cell lines, chemicals and antibodies

Human glioblastoma cell lines U87 and T98G were obtained from and grown in growth media recommended by the American Type Culture Collection. The human medulloblastoma cell line UW228, developed by Dr. Francis Ali-Osman (Duke University, NC), was cultured in Dulbecco’s modified Eagle’s medium (DMEM). All growth media were supplemented with 10% fetal bovine serum and antibiotics. Monoclonal antibodies to MGMT and actin were purchased from Millipore Corporation, whereas anti-cleaved caspase 3 and anti-cleaved poly ADP-ribose polymerase were purchased from Cell Signaling Technology. Anti-ALDH antibodies were from BD Biosciences. BG-polyethylene glycol (PEG)–biotin was a gift from Covalys Biosciences AG (Switzerland). DSF was purchased from CreCENT Chemical Company. Unless mentioned otherwise, all chemicals and reagents were purchased from Sigma–Aldrich Company.

Preparation of copper-chelated DSF

Copper-chelated disulfiram (CuDSF) was synthesized by mixing equimolar amounts of DSF and CuCl\textsubscript{2} for 24 h followed by extraction with chloroform as described previously (24). The final product was dried and stored in a desiccator.

Expression and purification of human recombinant MGMT protein

Hexa-histidine tagged human MGMT was expressed in Escherichia coli and purified as described by us previously (25).

Assay of MGMT activity

The DNA repair activity of MGMT was measured by the transfer of [\textsuperscript{3}H]-labeled methyl groups from the O\textsuperscript{6}-position of guanine in the DNA substrate to the MGMT protein as described previously (25,26). The DNA substrate enriched in O\textsuperscript{6}-methylguanine was prepared by reacting [\textsuperscript{3}H]-methyl-N-ethylnitrosourea (GE Healthcare, 60 Ci/mmol) (21). Briefly, the cell pellets were washed with cold phosphate-buffered saline (PBS), disrupted by sonication in the assay buffer (30 mM Tris–HCl pH 7.5, 0.5 mM dithiothreitol (DTT), 0.5 mM ethylenediaminetetraacetic acid (EDTA), 5% glycerol and 20 μg/mL spermidine) and centrifuged. The extracts (50–150 μg protein) were supplemented with the [\textsuperscript{3}H]-DNA (10,000 cpm) and incubated at 37°C. The reactions were terminated by the addition of trichloroacetic acid and the DNA substrate was hydrolyzed at 80°C, followed by filtration on glass fiber discs; the radioactivity present in protein precipitates was quantitated (25).

Western blotting

After trypsinization, the cell pellets were washed with cold PBS and subjected to sonication in 50 mM Tris–HCl (pH 8.0) containing 1% glycerol, 1 mM EDTA, 0.5 mM PMSF and 2 mM benzamidine and centrifuged. Equal protein amounts from different treatments were electrophoresed on 12% SDS–polyacrylamide gels. Proteins were electro-transferred to Immobilon-P membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (pH 8.0) containing 0.1% Tween 20 for 3 h and subsequently incubated with appropriate primary antibodies. Antigen–antibody complexes were visualized by enhanced chemiluminescence (Pierce Company). Band intensities were quantified using a Bio-Rad VersaDoc Imaging system.

Immunofluorescence analysis of MGMT expression

UW228 cells were cultured on sterile coverslips and treated with DSF for 12 h. The treated and untreated cells were fixed with 4% parformaldehyde for 20 min and washed with PBS. They were blocked with 3% bovine serum albumin containing 0.2% Triton X-100 for 3 h. Cells were incubated with the anti-MGMT antibody overnight at 4°C, washed thrice with PBS and incubated with Alexa Fluor-488 goat anti-mouse IgG (Invitrogen) for 1 h. Cells were counterstained with Hoechst to observe the nuclei, washed and mounted on slides. Images were acquired and quantitated using an Olympus IX 81 fluorescence microscope.

In vitro degradation of DSF–treated MGMT protein

Proteolysis assays in the presence of rabbit reticulocyte lysates (30 μl final vol.) were performed in Tris–HCl (30 mM; pH 8.0) containing 0.5 mM DTT, 4 mM MgCl\textsubscript{2}, 1 mM adenosine triphosphate (ATP), 1 μg ub, 0.7 μg recombinant MGMT (rMGMT) or 0.7 μg DSF treated rMGMT (15). DSF-exposed MGMT samples were dialyzed to remove the residual drug prior to the degradation assays. The reactions were initiated with the addition of 10 μl rabbit reticulocyte lysate (Promega), incubated at 37°C for 15–40 min, electrophoresed and immunoblotted using anti-MGMT antibodies. The protein bands were quantified by densitometry.

Cell cycle analysis

Sixty percent confluent cells were treated with 50 μM DSF for 12 h. This was followed by 100 μM BCNU. Untreated cells and cells treated with DSF and BCNU alone were used as controls. After the treatments, cells were allowed to grow for 24 and 48 h. At each of these time points cells were harvested and fixed in 70% ethanol. The cells were then washed with PBS and resuspended in the presence of RNase (1 μg/ml) and propidium iodide (PI, 50 μg/ml) for 30 min. Cell cycle histograms were generated using a BD Accuri C6 flow cytometer.

Assay for interstrand DNA cross-linking in tumor cells

UW228 cells pretreated with 50 μM DSF for 12 h were treated with melphalan (0–48 h, 100 μM) or BCNU (0–72 h, 100 μM). DNA from cells was isolated by lysis in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 0.5% SDS, and 100 μg/ml RNase A and proteinase K (1 mg/ml) digestion. DNA was precipitated with ethanol and dissolved in TE buffer. The drug-induced DNA cross-linking was measured by the ethidium bromide fluorescence assay as described previously (27). Briefly, 5–10 μg DNA was dissolved in assay buffer (20 mM potassium phosphate and 2 mM EDTA, pH 11.8) in duplicate. One set of tubes was heated at 100°C for 10 min and cooled to room temperature. Ethidium bromide was added to 1 μg/ml, and the fluorescence was measured (305 nm excitation and 585nm emission) using an LS-50 variable wavelength spectrophotofluorometer (Perkin Elmer). The fluorescence readings were used to compute the cross-link index as described earlier (27).

Cell survival assays

These were performed using the yellow tetrazolium dye [3-(4, 5-dimethyl-thiazolyl-2)-2, 5-diphenyl tetrazolium bromide] (MTT) as described previously (15). Cells (15 000 per well) in 24-well plates were treated with DSF (50 μM) for 12 h before exposure to the alkylating agents. The purple color developed due to formazan was measured at 570 nm using a SPECTRAFluor Plus plate reader (Tecan). Cell proliferation was also assessed by colony formation assays on soft agar (25). In these assays, UW228 cells treated with 50 μM DSF were suspended in media containing DMEM, 20% fetal bovine serum, 0.4% agarose, nonessential amino acids and BCNU (0–75 μM) or TMZ (0–750 μM). Ten thousand cells from these suspensions were layered on 3 ml of the same medium containing 0.6% agar in 60 mm culture plates. Seven to ten days after plating, cell colonies in triplicate plates were counted and cell survival computed.

Animal studies

Experiments with mice were conducted strictly in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Male CD1 mice were obtained from Charles River Laboratories, Wilmington, MA, and fed ad libitum. Mice weighing 19–21 g were administered a single intraperitoneal (i.p.) dose of 150 mg/kg DSF dissolved in 50 μl DMSO i.p. injections (six animals per group). Control mice received DMSO alone. The animals were killed at 1, 3, 6, 9 and 12 h after drug administration. Female, random bred, 4–6 week old, athymic (nu/nu) mice were obtained from Charles River Laboratories, Wilmington, MA. For xenograft studies, 7 × 10\textsuperscript{6} T98 human glioblastoma (T98G) cells were injected subcutaneously into the flank area of each animal, and within 6–8 days palpable tumors appeared. The animals were weighed and the tumors were measured twice weekly. When tumors were in the range of 300 mm\textsuperscript{3}, animals were treated with a single i.p. injection of 150 mg/kg DSF. Tissues and tumors were removed to analyze MGMT activity and protein levels at 3, 6, 9, 12, 18 and 24 h post DSF administration. The liver and tumor tissues were harvested, washed and homogenized in a buffer (50 mM Tris–HCl buffer (pH 8.0) containing 3% glycerol, 50 mM NaCl, 1 mM EDTA, 0.5 mM PMSF and 2 mM benzamidine and 0.5% Triton-X) using a Polytron. The lysates were centrifuged at 12 000 g and the resulting extracts were used for MGMT activity and western blot analyses.
**Statistical analysis**

All experiments including the western blotting and immunocytochemical analyses were performed four times independent of each other. Results were assessed by Student’s t-test. Significance was defined as *P* < 0.05. Power analysis was used to calculate the number of animals required to achieve a statistical power of >80%.

**Results**

**DSF, Cu-DSF and CuCl₂ + DSF treatments result in MGMT inhibition and loss of MGMT protein in human brain tumor cells**

Our search for drugs that can interact with protein-bound reactive cysteines led to DSF, and this study on MGMT was prompted by our previous findings that DSF can induce the degradation of key redox-sensitive proteins such as the p53 and NF-κB in tumor cells (15). The initial experiments involved the treatment of the rMGMT protein and UW228 cell-free extracts (which contained MGMT) with DSF and quantitation of the DNA repair activity. Although 25 µM DSF inhibited the purified MGMT by 40%, the repair activity in the extracts was curtailed by 75%, suggesting a direct effect of the compound on MGMT protein (Figure 1A). Next, the MGMT-proficient human brain tumor cell lines, T98G and UW228 were treated with DSF (0–500 µM) for 12 h and the MGMT activity was measured in the extracts therefrom. In both cell lines, MGMT activity was inhibited by DSF in a dose-dependent fashion with approximately 70% inhibition in cells exposed to 50 µM DSF. Western blot analysis showed a gradual loss of MGMT protein, consistent with the extent of inhibition after 12 h DSF treatment (Figure 1B). DSF binds copper with a great affinity (18), and the CuDSF was more potent in this property with 10–15 µM drug eliminating >90% of the MGMT activity and protein in UW228 cells after 12h treatment (Figure 1C). We also exposed UW228 cells to 0.1 µM CuCl₂ in combination with DSF; this schedule, however, resulted in modest inhibition of MGMT activity and protein levels (Figure 1D). These results confirm that DSF and its metabolites are capable of inactivating the MGMT protein and lead to its breakdown in human cancer cells.

To delineate the appropriate time for alkylating drug treatments following MGMT inhibition, next we studied the time course of MGMT depletion by these three interventions. The data obtained are shown in Figure 2. The presence of 50 µM DSF in the culture medium caused a maximal 75% inhibition of MGMT activity and protein at 12 h,
Disulfiram inhibition of MGMT

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which was maintained till 24 h (Figure 2A). In comparison, CuDSF, at a 5-fold lesser concentration (10 µM) than DSF, elicited a >90% inhibition of MGMT activity and protein during the same period (Figure 2B). Consistent with the results from Figure 1D, the combination of Cu and DSF was less potent, with only 40–50% inhibition (Figure 2C). Collectively, the findings in Figures 1 and 2 reveal that DSF is capable of rapidly inactivating the MGMT and maintain the inhibition to allow the induction of increased alkylation damage.

Immunofluorescence evidence for MGMT depletion by DSF

To confirm the data obtained by western blotting in Figures 1 and 2, we performed immunostaining of MGMT in UW228 cells treated with DSF. The representative photomicrographs in Figure 3A show that the antibody-specific staining for MGMT was markedly less in DSF-treated cells. To test whether the thiol linkages introduced by DSF on the protein are reversible, in some experiments, the DSF-treated cells were exposed to 10 mM DTT for 30 min prior to treatment with MGMT antibodies. The intensity of antibody staining did not change after DTT, indicating the non-reversibility of the modification. Further, DTT was unable to reverse the inhibition of MGMT activity by DSF (data not shown). Thus, unlike the mixed disulfides introduced in S-thiolation reactions (29), the DSF linkages introduced in the MGMT protein appear largely irreversible.

Cellular MGMT is regenerated much faster after DSF treatment compared with the BG exposure

MGMT performs a stoichiometric reaction to accomplish the DNA repair and is not recycled to its active form (1,2). Instead, the inactivated protein is degraded and fresh translation has to occur to restore the MGMT levels in cells. The rate and extent of this recovery is an important factor in the extent of damage introduced to the normal cell genomes such as the bone marrow stem cells. Therefore, we compared the MGMT depletion and repletion kinetics after DSF treatment with that by BG, an established inhibitor of MGMT, currently in clinical trials. In these experiments, the UW228 cells were incubated with 50 µM DSF or 50 µM BG for 12 h, and then the cultures were washed to remove the residual drugs followed by resuspension in drug-free media to allow MGMT regeneration. MGMT protein and activity levels were analyzed during the depletion and repletion (Figure 3B). Although BG resulted in 100% loss of MGMT activity, DSF produced a 75% inhibition at 12 h post-drug treatments (Figure 3B, right panel). However, the post-recovery of MGMT activity was very slow in BG-treated cultures compared with that in DSF treatment. Although the MGMT activity recovered to 25% of control levels at 48 h post-BG, it reached ~95% in case of post-DSF at the same interval (Figure 3B, right panel). MGMT protein levels as determined by western analyses during the depletion/repletion cycle were consistent with and fully reflected the DNA repair activity profiles (Figure 3B, left panel). These data suggest that DSF functions as a transient inhibitor of MGMT, and that DSF-inactivated MGMT may undergo an accelerated proteolysis compared with the benzylated MGMT protein. Since the longer MGMT suppression is likely to mediate a continued accumulation of alkylation DNA damage, our observations suggest that in contrast to BG, the DSF-induced short-term inhibition of MGMT is likely to be beneficial in rescuing the host tissues from continued genomic injury.
Evidence that the interaction of DSF with active site cysteine of MGMT leads to DNA repair inactivation

The human MGMT protein possesses five cysteine residues, of which the Cys145, which accepts the alkyl groups, is the most reactive (21). In the first approach, to test whether DSF reacts with cys145, we used a biotin-labeled BG probe, which binds specifically to this cysteine residue (30). Purified rMGMT protein was incubated with 50 µM DSF in 40 mM Tris–HCl, pH 8.0, 1 mM EDTA for 20 min at 37°C. BG–PEG–biotin (5 µM) was then added and incubations continued for 15 min. The samples were electrophoresed, blotted and probed with streptavidin–horseradish peroxidase to detect the protein-bound biotin (upper panel). The blot was reprobed with antibodies to MGMT (lower panel). These data clearly demonstrate that DSF modifies the active site cys145 of MGMT, which in turn inactivates the DNA repair function.

DSF-treated MGMT protein is prone to ATP-dependent proteasomal degradation both in vitro and in tumor cells

Since the MGMT inactivated by BG is degraded through ub-proteolysis (4), it was of interest to investigate the mode of elimination of DSF-modified MGMT protein. Therefore, first we compared the in vitro degradation of DSF-treated and untreated rMGMT protein in rabbit reticulocyte lysates, which are known to promote the proteasomal degradation (31). The control and DSF-modified MGMT proteins were incubated with reticulocyte lysates in the presence of Mg2++, ATP and ub at 37°C. The reaction mixtures were blotted and probed with MGMT antibodies. The resulting western blot and the densitometric quantitation of protein bands are shown in Figure 4A. The data show that the untreated rMGMT remained largely undegraded during the 40 min incubation, whereas the DSF-modified protein disappeared gradually, 30% at 10 min, 45% at 25 min and 70% at 40 min of incubation (Figure 4A). To show the involvement of the ub-proteolysis in cells, we used PS-341 (Bortezomib; Velcade), a clinically used proteasome inhibitor. UW228 cells were treated with PS-341 alone for
6h, DSF alone, exposed to DSF after PS-341, or co-incubated with PS-341 and DSF, and the lysates were processed for western blotting of MGMT. PS-341 co-incubation with DSF decreased the MGMT degradation by just 20%, whereas the cells preexposed to PS-341 and then to DSF showed a >50% reduction in the loss of MGMT protein (Figure 4B, lanes 4 and 5). Taken together, the results presented in Figure 4A and B confirm that DSF-modified MGMT is perceived as a structurally altered/damaged protein in cells and eliminated through the ub-proteasomal route.

**MGMT inhibition by DSF markedly increases the cross-linking of DNA induced by BCNU**

As discussed in the Introduction, the inhibition of MGMT-mediated DNA repair is expected to augment the levels of alkylation DNA damage and the interstrand-cross-linking of DNA induced by bifunctional alkylating agents. This hypothesis was tested by treating the UW228 cells with DSF for 12h followed by BCNU or melphalan as described in Materials and methods. An ethidium bromide fluorescence assay, well established in our laboratory, was used to determine the levels of cellular DNA cross-linking. Cells treated with DSF and BCNU showed an approximately 2-fold increase in interstrand cross-links as compared with BCNU alone (Figure 4C). However, DSF was unable to increase the cross-links generated by melphalan. Although BCNU introduces the chloroethyl groups to the O6-position of guanine, which are substrates for MGMT, melphalan is not an O6-guanine alkylator, but predominantly generates N7-guanine alkylations (32); thus, MGMT does not interfere with the damage induced by melphalan, and this explains the inability of DSF to enhance the melphalan-induced DNA cross-linking.

Fig. 4. Proteasomal degradation of DSF-modified MGMT. (A) In vitro degradation of DSF-treated rMGMT protein. 0.5 µg of unmodified rMGMT and DSF-treated rMGMT (after dialysis) proteins were subjected to ATP and Mg2+-dependent degradation in rabbit reticulocyte lysates for 0–40 min as described in Materials and methods. The samples were immunoblotted and probed with MGMT antibodies (right panel). The densitometric quantitation of protein bands on the western blots is shown as a line graph on the left. (B) The proteasome inhibitor PS-341 curtails the degradation of DSF-modified MGMT protein in brain tumor cells. UW228 cells were treated with 10 µM PS-341 alone for 6h (lane 2), with 50 µM DSF alone for 12h (lane 3), pretreated 6h with 10 µM PS-341 followed by 50 µM DSF for 12h (lane 4) and co-incubated with PS-341 and DSF for 6h (lane 5). Cell lysates were western blotted for the MGMT protein. (C) DSF increases the DNA damage induced by MGMT-targeted alkylating agents. Kinetics of DNA interstrand cross-links formed in UW228 cells after treatment with 100 µM BCNU or 100 µM melphalan is shown. Melphalan does not produce O6-alkylguanines (32) and served as a control. Cells were treated or untreated with 50 µM DSF for 12h to deplete the MGMT protein. They were then exposed to BCNU or melphalan. At times specified, the cells were harvested, DNA isolated and the extent of interstrand cross-linking of DNA was determined using the ethidium bromide fluorescence assay. Values are mean ± SD. The results were significant at P < 0.05. (D) G2/M blockade induced by BCNU is enhanced and extended in the presence of DSF. Histograms showing results of FACS-based cell cycle analysis of UW228 cells treated with BCNU or DSF ± BCNU. Asynchronous cultures were treated or untreated with DSF (50 µM for 12h) and then incubated with 100 µM BCNU. The cells were trypsinized at times indicated and analyzed by flow cytometry. (E) Enhanced levels of apoptosis markers—cleaved caspase 3 and cleaved poly ADP-ribose polymerase after DSF + BCNU treatments compared with BCNU alone.
MGMT inhibition by DSF enhances and prolongs the BCNU-induced G2/M cell cycle arrest in U22W2 cells

To determine the consequences of MGMT inactivation by DSF on cell cycle changes induced by BCNU, we performed flow cytometry (Figure 4D). U22W2 cells were treated with DSF alone, BCNU alone and a combination of DSF and BCNU, and histograms were generated from propidium iodide-stained cells. The results indicate that 50 μM DSF alone did not induce any cell cycle changes after 48 h. Treatment with BCNU, as expected (33), produced a significant G2/M blockade (33% of cells) at 24 h. Further, the cells pretreated with DSF and then exposed to BCNU showed a very strong accumulation of cells (~80%) in G2/M phase at 24 h, and this blockade was maintained at the same level at 48 h (Figure 4D).

In DSF-treated U22W2 cells, the greater level of DNA damage induced by BCNU (Figure 4C) and the resulting lengthy cell cycle arrest (Figure 4D) were associated with the activation of apoptotic machinery as reflected by an increased expression of cleaved caspase 3 and cleaved poly ADP-ribose polymerase proteins (Figure 4E). Collectively, the evidence provided so far distinctly indicates that the nontoxic drug DSF, acting through the inhibition of MGMT, is highly capable of potentiating the cytotoxic effects of alkylating agents.

Brief pretreatments with DSF sensitize MGMT proficient cells to alkylating agents

Others and we have shown previously that DSF can increase the efficacy of many anticancer drugs (15,34). In the context of this study, we performed cell survival assays using the MTT to determine the impact of DSF on alkylator-mediated tumor cell killing. DSF by itself, over a concentration range of 10–100 μM for 24 h, showed no toxic effects on U22W2 (Figure 5A) and other tumor cells (data not shown). Based on this, we choose 50 μM DSF preincubation for 12 h to test the potentiating of alkylating drugs. In this setting, TMZ (0–750 μM) + DSF combination showed a 4- to 5-fold increased cytotoxicity compared with TMZ alone (Figure 5B). For BCNU, the potentiation was 3-fold (Figure 5C). To confirm the MGMT-specific effects of DSF in cell survival assays, we used two types of controls. First, the drug melphalan, which does not generate O6-alkylguanines (35), was used and DSF did not increase the melphalan-induced cell killing (Figure 5D). Second, the effects of TMZ and BCNU were tested in DSF-pretreated U87 malignant glioma cells, which lack MGMT expression due to promoter methylation (36). DSF did not increase the extent of cell killing by TMZ or BCNU in U87 cells (Figure 5E and F).

Colonony formation assays on soft agar were also carried out to confirm the DSF-induced increases in tumor cell killing. DSF treatment of U22W2 cells followed by BCNU or TMZ exposures resulted in a 3-fold enhanced cytotoxicity for both drugs (Figure 5G). The cell survival assays, again, demonstrate that MGMT inhibition by DSF is a significant factor in amplifying the cytotoxic effects of the clinically used alkylating agents.

DSF administration attenuates MGMT activity and protein levels in liver and brain tissues in normal mice

To validate our findings of MGMT inhibition by DSF in a preclinical setting, we administered a single dose of DSF (150 mg/kg, i.p.) to CD1 mice, isolated the liver and brain tissues 1, 3, 6, 9 and 12 h post-injection, and determined MGMT activity in clarified tissue lysates. MGMT activity in six animals at each time point was averaged and used to compute the changes in DNA repair activity. There was a gradual reduction of MGMT in brain with a 40% decrease of the repair activity occurring at 9–12 h (Figure 6A, upper panel). The MGMT protein levels correlated well with the observed changes in the activity (Figure 6A, lower panel). In liver tissues, MGMT inhibition was much stronger with a 60% decrease observed at 12 h relative to the controls (Figure 6B, upper panel). Hepatic MGMT protein appeared to undergo degradation in DSF-treated animals, in a manner similar to that observed in cancer cell lines (Figure 6B, lower panel). Significantly, the hepatic levels of ALDH protein, the primary target of DSF mediating the alcohol antagonism, also showed a progressive decline in DSF-treated animals, much similar to that observed for the MGMT protein. To our knowledge, this is the first report showing a decrease of ALDH protein in DSF-treated animals. Overall, the data obtained in animal studies establish that DSF can curtail the MGMT activity in the brain and strengthens the drug’s utility in brain tumor therapy.

Selective depletion and repletion of MGMT in T98 glioblastoma xenografts after DSF administration

To explore the MGMT inhibition kinetics as well as its regeneration pattern in human cancers following DSF intake, we established an ectopic xenograft model by injecting the T98G cells in nude mice. The tumor-bearing animals were given a single dose of DSF (150 mg/kg), and MGMT activity and protein levels were measured in the tumor and liver tissues during 3–24 h period. As shown in Figure 6C and D, both the hepatic and cancer tissues displayed significant down-regulation of the MGMT. However, the T98G tumors showed higher levels of inhibition relative to the liver. Thus, there was a 50% inhibition of MGMT activity in the tumor at 9 h post-DSF compared with 29% reduction in the liver. Despite this difference, the DNA repair activity repleted to control levels by 24 h in both tissues (Figure 6C and D, left panels). Consistent with its stoichiometric mechanism, the MGMT protein levels during this degradation and regeneration phases correlated well with the extent of DNA repair activity in these tissues. Our data confirm the ability of DSF to curtail and degrade the MGMT protein in tumor tissues and the transient nature of inhibition.

Discussion

DSF is a drug discovered and developed in the 19th century that inhibits the ALDH and reduces ethanol consumption in alcoholics. Besides the alcohol deterrent activity, DSF has been known for over 40 years to have significant anticancer effects. Recently, there has been a renewed interest in repurposing the DSF for cancer chemotherapy (37). The long list anticancer effects of DSF include the inhibition of (i) P-glycoprotein; (ii) NF-kB; (iii) metalloproteinases; (iv) invasion and angiogenesis; (v) proteasome and (vi) tumor growth in various xenograft settings (38,39). The antineoplastic effects of DSF have been variously attributed to the redox changes, permeabilization of the mitochondrial membrane, inactivation of Cu/Zn superoxide dismutases, formation of mixed disulfides with proteins and inhibition of cell signaling (40). The usefulness of DSF for primary brain cancers has been suggested (41,42), though not fully explored, despite the known ability of the drug to cross the blood–brain barrier (43,44).

A major finding of this study is the demonstration of DSF as a direct and mechanism-based inhibitor of the human DNA repair protein MGMT. However, a number of enzymes acting on alkylation damage such as the APE1 and topoisomerase III possess reactive cysteines (45,46) and are likely to be modified and inactivated by DSF as well. The incidence of brain tumors in adults and pediatric patients has seen a continued increase in recent years with an estimated 17 000 new cases diagnosed and 12 000 death every year in the USA (47,48). Brain tumors are also the second leading cause of cancer deaths among pediatric patients, just next to leukemia (49). Gliomas, medulloblastomas and other brain cancers remain the most therapeutically challenging and chemotherapy using hydrophobic alkylating drugs are the mainstay in their treatment (50). The clinically used alkylating agents also sensitize the brain tumors to radiation (51), and therefore there is a great need for strengthening/improving the efficacy of the monofunctional and bifunctional alkylating drugs used for central nervous system cancers. DSF has been shown to increase the cytotoxicity of many anticancer drugs such as the cisplatin, gemcitabine, paclitaxel and 5-fluorouracil through unknown mechanisms (35). Our studies help to add the MGMT-targeted alkylating agents (triazenes, chloroethyl-nitrosoureas and others) to this list and explain the direct modification of MGMT protein by DSF as the mechanism underlying the drug potentiation.
Recently, as a part of an international effort, we proposed that a cocktail of nine pharmacologically well-characterized nontoxic drugs, collectively called CUSP9, be added to a continuous low dose TMZ for improving survival and quality of life for relapsed glioblastoma patients (52), who have one of the worst 5 year survival rates among all human cancers. The drugs suggested include artemunate, auranofin, DSF, copper gluconate, nelfinavir and others (52). Of these, DSF has the strongest evidence of potential benefit in central nervous system cancers. The copper gluconate in the regimen is meant to enhance the efficacy of DSF, because the copper-bound drug has been shown to be more potent and exert higher levels of cytotoxicity (24,38). In fact, the ongoing clinical trials of DSF in metastatic cancers of the liver, prostate and melanoma include the copper gluconate or zinc gluconate as a component (53). Consistent with this premise, we found that CuDSF was about 5-fold more potent than DSF in inhibiting the MGMT activity in cultured brain tumor cells (Figure 1B and C).

The important findings of our study are summarized in Figure 6E. Chemically, DSF has a symmetrical structure and its first metabolic step is the reduction of the disulfide group at the center of the molecule to yield two diethyldithiocarbamate moieties. Diethyldithiocarbamate is a potent inhibitor of ALDH, forming mixed disulfide bridges with a critical cysteine near the active site (13). Our data from the cell culture and animal studies indicate that DSF interacts with Cys145 of MGMT the same way to inactivate the DNA repair protein (Figure 6E). We postulate that the covalent adducts induced by DSF alter the secondary and higher order structure of the MGMT protein, allowing the inactivated protein to be recognized by the ubiquitination-dependent proteolysis. Evidence clearly pointing to the involvement of the ubiquitination-dependent proteolysis in the processing of DSF-conjugated MGMT was obtained (Figure 4A and B). The earlier findings that DSF mediates the degradation of several redox-sensitive proteins (p53, NF-kB, ubiquitinating enzyme E1)
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In tumor cells clearly agree with the observations made here. The covalent adduct introduced by DSF is non-reducible at physiological concentrations of GSH, and actually, the endogenous GSH has been reported to increase the inactivation of ALDH by DSF metabolites (54). Similarly, we found that MGMT inactivation by DSF was not reversible by dithiothreitol or GSH (data not shown).

A unique outcome of this study is that DSF has MGMT-dependent biochemical effects. Evidence from literature clearly shows that DSF targets several regulatory proteins/signaling pathways and promotes the efficacy of various anticancer drugs in a pleiotropic ‘MGMT-independent’ manner as well. We showed potent inhibition of MGMT activity and the resulting protein degradation in tumor cell lines and animal tissues. BG, the MGMT inhibitor in clinical trials, has also been shown to inhibit MGMT in normal tissues in animals and humans (55,56). DSF, in contrast to BG, appeared to be a weaker inhibitor of MGMT causing a short-term inactivation of MGMT >30h, which is still sufficient for enhancing the efficacy of alkylating agents, because the alkylation of DNA is a rapid process. The promotion of BCNU-induced DNA cross-linking in DSF-treated cells (Figure 4C) again supports this postulate. Further, we noted a preferential inhibition of tumor MGMT activity and its depletion in T98G xenografts (Figure 6D). It is notable that a number of xenograft and patient studies have consistently linked such an attenuation of MGMT content in malignant tissues to an improved therapeutic outcome (57–59). The faster regeneration of MGMT we observed in cell culture (Figure 3B) and mouse liver (Figure 6) following DSF treatment is likely to decrease the toxicity to the normal tissues by alkylation damage.

From the discussion above, it is clear that DSF has a huge potential for therapy of central nervous system tumors. It is a drug already...
approved by regulatory agencies for human use, and its pharmacoki-
netics and safety issues are well known; this should enable a faster
clinical application. DSF has a favorable lipophilic profile (mole-
cular weight 296.5 Daltons, partition coefficient Log P of 2.8) to cross
the blood–brain barrier, and many studies have established its entry
into the brain (43,44). Further, DSF administered in the absence of
alcohol is largely nontoxic and tolerated very well. Nevertheless,
whether DSF has a similar affinity to MGMT and ALDH is unclear.
As such, the potency and extent of MGMT inhibition in human tis-
ues by DSF remain to be determined. Although the routine dosage
of DSF in alcoholic patients is 100–200 mg daily up to 2 weeks, it
can be given in higher amounts (800 mg/day) in a non-alcoholic adju-
vant setting (60). With a long half-life in the range of 6–11 h reported
for this drug (13,54), a significant inhibition of MGMT is possible.
In rats, DSF at high doses of 600 mg/kg daily for 3 days or smaller
doses (up to 100 mg/kg) at twice a week dosing has been shown not
to exert any liver toxicity (54). Also noteworthy is that DSF appears
to be selectively toxic to human cancer cells compared with the normal
cell counterparts; thus, in pairs of the chronic lymphocytic leukemia
and normal lymphocytes (61), invasive cancer and normal endothe-
rial cells (62), and the glioblastoma and normal astrocytes (63), there
was a preferential killing of cancer cells by DSF. In summary, we
exploited the redox-sensitive nature of the human MGMT protein,
and since DSF (i) is nontoxic; (ii) is hydrophobic enough to cross
the blood–brain barrier; (iii) is unlikely to induce tumor resistance and
(iv) has MGMT-independent signaling effects that may actually pro-
 mote the chemotherapy, we believe that the ability of DSF to create
an MGMT-deficient state holds excellent promise for improving the
brain tumor therapy.

Besides ethanol, the ALDHs metabolize several xenobiotic and endog-
 enous compounds such as the retinoic acid and γ-aminobutyric acid
(64). The acetaldehyde and other aldehydes generated in these reactions
can also inhibit the MGMT (9); however, the innate production of such
compounds is likely to be very low (65). A recent report also linked the
increased expression of ALDH 1A1 to TMZ resistance (66), although the
mechanism involved is unknown. In contrast to the enhanced anticancer
effects, our observations suggest that chronic alcohols undergoing DSF
therapy for a long time are likely to have an increased risk for devel-
oping cancer. This is because alcohol by itself is known to downregu-
late the MGMT activity (8–10). Through a direct effect reported here,
DSF can further exacerbate the MGMT inactivation, and the ability of
different organs to defend themselves against the endogenous and envi-
ronmentally derived alkylating agents may be compromised. Such an
unrepaired DNA damage, particularly in the regulatory oncogenes and
tumor suppressor genes, may manifest in harmful mutations and promote
the genomic instability. The reported occurrence of frequent mutations
in K-ras, p53 and β-catenin genes in cells with reduced MGMT activity
or silencing of the MGMT gene through promoter methylation in human
cancers (67,68) is consistent with this premise.

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References

by O6-methylguanine. Mutagenesis, 17, 483–487.
4. Srivengup, K.S. et al. (1996) Ubiquitination-dependent proteolysis of O6-
methylguanine-DNA methyltransferase in human and murine tumor cells
following inactivation with O6-benzylguanine or 1,3-bis(2-chloroethyl)1-
in tissues of methionine-cysteine-deficient subcutant and adult mice. Comp.
6. Rydberg,B. et al. (1982) Nonenzymatic methylation of DNA by the intra-
cellular methyl group donor S-adenosyl-L-methionine is a potentially
mutagenic reaction. EMBO J., 1, 211–216.
 tion, alkylation, and repair of DNA from hepatocytes and nonparenchymal
cells following dimethylhydrazine administration. Carcinogenesis, 3,
1293–1297.
8. Wilson,D.M. et al. (1994) Acute ethanol exposure suppresses the repair of
O6-methylguanine DNA lesions in castrated adult male rats. Alcohol Clin.
9. Espina,N. et al. (1988) In vitro and in vivo inhibitory effect of ethanol and
acetaldehyde on O6-methylguanine transferase. Carcinogenesis, 9,
761–766.
of hepatic O6-methylguanine DNA methyltransferase in chronic liver dis-
enzyme E1 by N,N-diethyldithiocarbamate inhibits ubiquitin activation
and is accompanied by striatal injury in the rat. Chem. Res. Toxicol.,
25, 2310–2321.
redox-sensitive proteins by thiol-conjugating and -nitrosylating drugs in
17. Kaina,B. et al. (2010) Targeting O6-methylguanine-DNA methyltrans-
erase with specific inhibitors as a strategy in cancer therapy. Cell. Mol.
18. Rabik,C.A. et al. (2006) Inactivation of O6-alkylguanine DNA alkyl-
transferase as a means to enhance chemotherapy. Cancer Treat. Rev.,
32, 261–276.
DNA methyltransferase in human tumor cells by O6-benzylguanine in
MGMTP140K protects human CD34+ cells against the combined toxicity
of O6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea or temozolo-
pKa and high reactivity of cysteine 145. Biochemistry, 42, 10965–10970.
22. Niture,S.K. et al. (2005) Human MGMT is a prime target for inactivation
by oxidative stress, mediated by glutathionylation and oxidation of the
23. Liu,L. et al. (2002) Inactivation and degradation of O(6)-alkylguanine-
DNA alkyltransferase after reaction with nitric oxide. Cancer Res.,
62, 3073–3083.
25. Srivengup, K.S. et al. (2000) Protein phosphorylation is a regulatory
mechanism for O6-alkylguanine-DNA alkyltransferase in human brain
methyltransferase activity and its application to human neoplastic and non-
27. Ali-Osman,F. et al. (1995) Formation and repair of 1,3-bis-(2-chloroethyl)1-
nitrosourea and cisplatin induced total genomic DNA interstrand
used to assess survival or proliferation of mammalian Cells. Proc. West.
analysis of redox status of protein cysteines by glutathione-affinity chro-

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